On the origin of pontocerebellar hypoplasia: Finding genes for a rare disease
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Een goed begin is het halve werk
Maar een goed begin is maar de helft

- De Jeugd van Tegenwoordig
INTRODUCTION

PONTOCEREBELLAR HYPOPLASIA FROM A CLINICAL AND GENETIC PERSPECTIVE

Veerle RC Eggens
The History of Pontocerebellar Hypoplasia

It sounds simple: the main characteristic of pontocerebellar hypoplasia is pontocerebellar hypoplasia. In this chapter we will describe the differences between the various types of pontocerebellar hypoplasia (PCH) and we will set out the past and present state of clinical and genetic aspects of PCH research.

The first description of a patient with a hypoplastic pons and cerebellum was published in the beginning of the 20th century [1]. In 1929, Krause associated clinical features to this pathology [2]. He reported a child of 16 months old with swallowing problems, spasticity and microcephaly. Pathological investigation revealed a cerebellum severely diminished in size, while gross formation of cortical gyri and sulci was intact.

In the last two decades much progress has been made in research on pontocerebellar hypoplasia (PCH). A big step was the identification of the causative gene for PCH in a cluster of related families in Volendam, a genetic isolate in the Netherlands. In 1990, Barth described seven children of five related families in this area, presenting microcephaly, spastic paresis and extrapyramidal dyskinesia [3]. CT scans revealed severe pontocerebellar hypoplasia and cerebral atrophy. Histologically, loss of neurons was more evident in the pons and the cerebellum compared to other brain regions. PCH was initially classified in two subtypes: PCH with (subtype 1) or without degeneration of the motor neurons in the anterior horn of the spinal cord (subtype 2). Nowadays, the number of PCH subtypes is extended to ten, based on clinical and genetic features. Shared hallmarks in all subtypes include hypoplasia and/or atrophy of the cerebellum and pons, an early - in most cases fetal - onset of the disease, severe developmental delay and very limited cognitive and motor skills. The small volume of the pons and the cerebellum is mainly due to loss of Purkinje cells, fragmentation of the dentate nucleus and loss of pontine nuclei. Impaired foliation of the cerebellar hemispheres is common [4]. Currently, there is no cure for PCH. Treatment is only symptomatic and includes percutaneous endoscopic gastronomy feeding, respiratory support, treatment of dystonia and seizures and physiotherapy [5]. Age of death ranges from neonatal to late twenties, though most patients die in childhood.

PCH Subtypes - Clinics and Genetics

The PCH subtypes share common features, but each subtype has distinct clinical and genetic characteristics, which will be briefly discussed below (Figure 1.2 and Table 1.1). Pontocerebellar hypoplasia is an autosomal recessive disease. Due to the developments in genetics, especially the advantages in next generation sequencing,
the identification of new genes involved in rare diseases as PCH became less time consuming. Until a decade ago, linkage analysis in large families was the standard way of identifying a disease locus. Nowadays, exome or genome sequencing of “trios” consisting of a patient and his or her parents can be sufficient to identify novel disease genes. This development has boosted PCH research, and currently ten forms of PCH are defined. In the majority of patients the genetic background is known, but still a large cohort remains in which the genetic component of the disease is not yet revealed.

**PCH1** (OMIM 607596, 606489, 616081) is characterized by pontocerebellar hypoplasia plus degeneration of motor neurons in the anterior horn of the spinal cord. Initially, PCH1 was associated with death within the first year of life [6]. In recent years, it has become clear that the PCH1 phenotype is much broader, with possible survival into puberty [7]. A subset of patients shows an intact pons, and the level of cerebellar hypoplasia is variable (Chapter 3 of this thesis, [8]). Patients suffer from hypotonia and severe developmental delay. Some patients are able to walk or sit independently, but lose this ability as disease progresses [9].

Mutations in the **EXOSC3** gene are found in about half of all PCH1 patients [10]. This gene encodes component 3 of the exosome complex, a complex with exoribonuclease activity that is involved in various RNA processing and degradation processes [11]. In three families with PCH1, mutations in the vaccinia-related kinase 1 (**VRK1**) have been found [12,13]. VRK1 is a kinase thought to be involved in cell proliferation, cell cycle and carcinogenesis [14] and regulation of Cajal bodies [15]. Recently, evidence for a neuronal function for VRK1 was presented, as Vrk1 knockdown in mice impairs migration of cortical neurons and affects the cell cycle of neuronal progenitors [16]. In one family classified as PCH1, mutations in the **TSEN54** gene were identified [17], see below. Finally, mutations in **EXOSC8**, encoding component 8 of the exosome complex, cause a PCH1-like disease, including degeneration of oligodendroglia cells [18].

**PCH2** (OMIM 277470, 612389, 612390, 613811) is the most common and therefore best-studied subtype of pontocerebellar hypoplasia. The first reports by Barth [3] describe patients with this subtype. A minority of the patients shows atrophy of the cerebral cortex on MRI [19]. Seizures are often reported in this subtype, as well as swallowing problems, dyskinesia, sleep disorders and gastroesophageal reflux. The majority of patients can fix and follow, grasp objects and have social smile. Life expectancy ranges from infancy to early puberty [20].

The first gene for PCH was identified in the Volendam PCH2 cohort. A founder mutation in the transfer-RNA (tRNA) splicing endonuclease (**TSEN**) complex was identified [21]. This variant (c.919G>T, p.A307S) lies in one of the subunits of the TSEN complex (**TSEN54**) and is the most common mutation in PCH2. The p.A307S mutation is highly prevalent in Volendam, with a carrier frequency of 14.2% [22] and is also seen in
other (Caucasian) populations. Patients with the common mutation have a typical dragonfly-like pattern of the cerebellar hemispheres on coronal brain MRI, where the vermis is relatively intact. Besides the p.A307S mutation, a number of other missense mutations in TSEN54 have been found to underlie PCH2. In a few isolated families mutations in TSEN2 and TSEN34 - other subunits of the TSEN complex - have been identified [21].

**PCH3** (OMIM 608027) is described in only a few families [23,24]. Patients show similar symptoms as PCH2 patients, although without extrapyramidal involvement. All but one patient had optic atrophy. PCH3 has also been described in combination with the cardiac malformation syndrome tetralogy of Fallot [25] and Vitamin A deficiency [26,27]. Recently, a homozygous nonsense mutation in piccolo presynaptic cytomatrix protein (**PCLO***) was identified in an Omani family with PCH3 [28]. PCLO is potentially involved in regulation of presynaptic proteins and vesicles.

For a long time, **PCH4** (OMIM 225753) and **PCH5** (OMIM 610204) were considered to be distinctive subtypes, based on neuroradiological aspects. In the few families with PCH5 the vermis was supposed to be more affected than the hemispheres, while these structures were equally affected in PCH4 [29]. Reconsideration of the phenotypes and genetics of the two subtypes led to the conclusion that separation of the two types is dispensable. Patients with PCH4 or 5 present the same features as PCH2 patients, although with a more severe and earlier onset. Clonus, contractures, hypoventilation and hypoplasia of the cerebrum are more often seen in PCH4 or 5 than in PCH2. Also, C-shaped inferior olives indicate a very early prenatal onset of the disease. Most patients with PCH4 or 5 die during infancy [19].

The more severe clinical presentation of PCH4 or 5 in comparison to PCH2 is reflected in the underlying genetics. Whereas both subtypes can be caused by mutations in **TSEN54**, PCH2 is due to missense mutations and PCH4 and 5 are due to compound mutations including one heterozygous missense mutation and one functional null allele [19].

**PCH6** (OMIM 611523) is rare and combines PCH with mitochondrial respiratory chain defects, manifested in elevated lactate levels. Degeneration of the cerebellum and cerebrum is very progressive in patients with PCH6. Autopsy on two siblings with PCH6 revealed immature cerebella, similar to a developmental stage of less than 18 weeks, and immature simplified cortical gyri [31]. Most patients survive into childhood [30].

Several missense mutations in the **RARS2** gene have been identified in patients with PCH6 [32]. RARS2 encodes for the nuclear encoded mitochondrial arginyl-tRNA synthetase, which connects the amino acid arginyl to its corresponding mitochondrial tRNA.
Patients with **PCH7** (OMIM 614969) have both brain abnormalities and genital abnormalities. For years it was uncertain whether PCH7 was an isolated disorder or a coincidence of two disorders [33-35]. Recently, we described eight families with PCH7, all with mutations in the target of Egr1 (*TOE1*) gene, supporting that PCH7 is an isolated disease (Chapter 2 of this thesis). Brain MRI shows a hypoplastic pons and cerebellum, large ventricles and thin white matter. Patients suffer from axial hypotonia, hypertonic limbs, seizures and severely delayed development. All 46, XY patients have ambiguous genitals and one 46, XX patient had atrophic ovaries and absent menarche at the age of 20 years. Survival range is broad; three patients died at the age of 24 weeks, 2 years and 3 years respectively, while two siblings in their twenties are still alive.

*TOE1* is supposed to have various functions, as it is involved in the cell cycle [36], exhibits deadenylation activity [37] and is essential for the maintenance of Cajal bodies [38]. Cajal bodies are nuclear entities that include splicing factors, therefore *TOE1* might be involved in mRNA splicing.

**PCH8** (OMIM 614961) is described in three families from Peru and Puerto Rico [39] and caused by mutations in charged multivesicular body protein 1A (*CHMP1A*). It is thought to be a non-progressive form of PCH, including microcephaly, ocular abnormalities, hypoplasia of pons and cerebellum, reduced cerebral white matter and thin corpus callosum. Some patients could sit or walk independently. The developmental rather than degenerative nature of PCH8 is reflected in the function of the affected gene; *CHMP1A* is involved in cell proliferation and chromatin modelling.

**PCH9** (OMIM 615809) is described in five families [40]. Distinctive for this subtype is the ‘figure 8’ appearance of the brain stem on axial MRI images and corpus callosum hypoplasia. Patients present progressive microcephaly, impaired swallowing, spasticity and absent gross and fine motor skills.

Patients with mutations in the *AMPD2* gene have been characterised as PCH9 patients. The gene encodes for adenosine monophosphatase deaminase 2 and is essential in the maintenance of cellular guanine nucleotide levels. A reduction of guanine levels, due to mutations in this gene may lead to a defect in protein translation initiation.

**PCH10** (OMIM 615803) is the most recently described subtype of PCH and was initially identified in nine Turkish families (Chapter 4 of this thesis, [41,42]). Patients suffer from both central and peripheral nervous system abnormalities. Brain MRI shows small pons, cerebellum and brainstem, as well as cortical involvement.

Mutations in cleavage and poly-adenylation factor 1 (*CLP1*) account for PCH10 cases. All nine families harboured a homozygous missense mutation (c.419C>A, p.R140H) in this gene. *CLP1* is associated with the TSEN complex and involved in tRNA processing [43].
Figure 1.1: Brain MRI of PCH1-10. A control MRI is shown in A. B presents a PCH1 patient with a p.D132A mutation in EXOSC3 [44]. A typically unaffected pons is seen. C shows a PCH2 patient with a homozygous p.A307S mutation in TSEN54 [4]. D is a PCH3 patient with a PCLO mutation [28]. E is an example of a PCH4 patient with cortical atrophy [4]. F presents a PCH6 patient with a RARS2 mutation [32]. G presents a PCH7 patient; characteristically large ventricles and thin corpus callosum are present. Corpus callosum atrophy can also be appreciated in H (PCH8; [39]), I (PCH9; [40]) and J (PCH10; [41]).

Table 1.1: Clinical, radiological and genetic features of PCH subtypes.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Distinctive clinical features</th>
<th>Anatomic features</th>
<th>Genetic features</th>
<th>Key references</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCH1</td>
<td>Anterior horn cell degeneration</td>
<td>Pons not always affected</td>
<td>EXOSC3 in majority of cases; TSEN54, RARS2, VRK1 in few families, EXOSC8</td>
<td>[7,8,12,13,17,18,45,46]</td>
</tr>
<tr>
<td>PCH2</td>
<td>Extrapyramidal symptoms</td>
<td>Dragonfly shaped cerebellar hemispheres in case of p.A307S mutation</td>
<td>TSEN54, TSEN2, TSEN34</td>
<td>[19,21,47,48]</td>
</tr>
<tr>
<td>PCH3</td>
<td>No extrapyramidal symptoms, optic atrophy</td>
<td>Variable</td>
<td>PCLO</td>
<td>[23-28]</td>
</tr>
<tr>
<td>PCH4 or 5</td>
<td>Severe PCH2</td>
<td>Often cortical atrophy, immature folia</td>
<td>TSEN54</td>
<td>[19,21]</td>
</tr>
<tr>
<td>PCH6</td>
<td>Mitochondrial respiratory chain defects</td>
<td>Neocortical atrophy</td>
<td>RARS2</td>
<td>[30-32]</td>
</tr>
<tr>
<td>PCH7</td>
<td>Disorders of sex development</td>
<td>Large ventricles, thin corpus callosum</td>
<td>TOE1</td>
<td>Chapter 2 of this thesis, [33-35]</td>
</tr>
<tr>
<td>PCH8</td>
<td>Non-progressive</td>
<td>Thin corpus callosum</td>
<td>CHMP1A</td>
<td>[39]</td>
</tr>
<tr>
<td>PCH9</td>
<td>'Figure 8' appearance of brain stem</td>
<td>'Figure 8' appearance of brain stem</td>
<td>AMPD2</td>
<td>[40]</td>
</tr>
<tr>
<td>PCH10</td>
<td>Both central and peripheral nervous system abnormalities</td>
<td>Relatively mild cerebellar hypoplasia</td>
<td>CLP1</td>
<td>[41,42]</td>
</tr>
</tbody>
</table>
Differential Diagnosis

In the diagnosis of PCH, a small pons and cerebellum on MRI is the main criterion. The degree of hypoplasia varies, making it a broad non-conclusive criterion, which is found in many other diseases. Cerebellar hypoplasia is often not an isolated symptom, but is accompanied by involvement of the cortex, motor neurons and/or white matter. Another distinction that can be made within the spectrum of early-onset cerebellar hypoplasias is the origin of the reduced size of the cerebellum; this can be a developmental defect, it can be neurodegenerative or a combination of both. The latter is thought to be the case in most patients: neurodegeneration sets in before the cerebellum is fully developed. Only PCH8 seems to be a non-progressive developmental form of PCH. Since the knowledge of genetics behind these conditions is growing fast, genetic testing can make the final diagnosis of a patient with cerebellar hypoplasia. Diseases that present overlapping features with PCH are briefly discussed below. Discriminating and shared features of these diseases and the various PCH subtypes are displayed in Figure 1.2.

A disorder that is very closely related to PCH is mental retardation and microcephaly with pontine and cerebellar hypoplasia (MICPCH; OMIM 300749), caused by mutations in the X-linked gene CASK. Both males and females can be affected, although the clinical outcome is more severe in males. MICPCH includes microcephaly, neocortical dysplasia with a simplified gyral pattern in some cases, pontocerebellar hypoplasia, and a thin brain stem. Patients have severe or profound intellectual disability [49].

In progressive cerebello-cerebral atrophy (PCCA, OMIM 613811) biallelic mutations in SEPSECS are found, resulting in the defective synthesis of the seleno-cysteine carrying tRNA [50]. Clinical and MRI findings closely resemble those of mild PCH2 [4].

Congenital disorders of glycosylation type 1A (CDG1A; OMIM 212065) overlaps with PCH in developmental delay, hypotonia and cerebellar dysfunction. Common additional symptoms in CDG1A are hepatic dysfunction, hypogonadism, and abnormal subcutaneous fat pads. CDG1A is caused by biallelic mutations in PMM2 [51].

A critical pathway in neuronal migration in the cortex and cerebellum is the RELN signalling pathway [52]. Two disorders are known in which this pathway is disrupted. Mutations in RELN can cause lissencephaly with cerebellar hypoplasia (OMIM 257320; [53]) and mutations in the VLDLR gene are associated with cerebellar hypoplasia, ataxia, mental disability and simplified cortical gyri (OMIM 224050). Clinical features are thought to be non-progressive [54].

Other non-progressive congenital cerebellar malformations are Dandy-Walker syndrome (OMIM 220200) and the various subtypes of Joubert syndrome. Typical for Dandy-Walker syndrome is a large posterior fossa with the hypoplastic cerebellum.
rotated upwards [55]. A characteristic image on axial MRI in Joubert syndrome is a molar tooth shaped pons and brain stem [56].

In spinocerebellar ataxias (SCAs) mental impairment is mild or absent. Importantly, cerebellar atrophy rather than hypoplasia is found, which is in line with the often late onset of this disease [57].

Also in spinal muscular atrophy type (SMA) cognitive function is usually not impaired. Brain MRI is normal, but EMG reveals denervation and muscle biopsy shows grouped atrophy. SMA type I (OMIM 253300) overlaps with most PCH types considering the early onset (birth – 6 months), muscle weakness and lack of motor development. SMA I is caused by biallelic pathogenic variants in SMN1 [58].

In the majority of cases, pontocerebellar hypoplasia arises due to genetic mutations. However, it is important to realise that prematurity (<32 weeks) is a risk factor for impaired cerebellar development [59,60].

![Figure 1.2: Pathological aspects of PCH subtypes and related disorders. Cerebellar hypoplasia, motor neuron degeneration, cortical atrophy, white matter abnormalities and neurodegeneration play part in the clinical manifestation of PCH and related early-onset brain disorders.](image)

**DISEASE MECHANISM**

Many of the genes involved in PCH play a role in RNA processing, and tRNA processing in particular. tRNAs guide amino acids to the ribosomes for protein synthesis and are therefore essential molecules in all cells of the body. Every amino acid is coded by multiple codons and for each codon multiple tRNA genes exist. Humans have 506 tRNA genes, of which six percent contains an intron. Some tRNA species are encoded solely by genes without an intron, for example the tRNA genes coding for tRNA-Ser. Other tRNA species are encoded by almost exclusively intron-containing genes. For
instance, thirteen out of fourteen tRNA-Tyr (gTa) genes contain an intron and all five tRNA-Ile (TaT) genes (Table 1.2).

Unlike mRNA, introns of a tRNAs are spliced out by a specialised splicing machinery, the TSEN complex. As discussed above, this enzyme complex is affected in the majority of PCH2 and PCH4 or 5 patients. The complex consists of two structural subunits (TSEN54 and TSEN15) and two catalytic subunits (TSEN2 and TSEN15) [61]. Cleavage of the tRNA gene results in a 5' and a 3' exon, which have to be ligated together. In vertebrates, two pathways of tRNA ligation exist, the “yeast-like” and the “animal-like” pathway (Figure 1.3). In the yeast-like pathway, the OH group on the 5' end of the 3'

<table>
<thead>
<tr>
<th>tRNA species - anticodon</th>
<th>Number of tRNA genes with an intron</th>
<th>Number of tRNA genes without an intron</th>
<th>Percentage of tRNA genes with an intron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-AGG</td>
<td>1</td>
<td>9</td>
<td>10.0</td>
</tr>
<tr>
<td>Arg-TCT</td>
<td>5</td>
<td>1</td>
<td>83.3</td>
</tr>
<tr>
<td>Leu-CAA</td>
<td>5</td>
<td>2</td>
<td>71.4</td>
</tr>
<tr>
<td>Ile-TAT</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Tyr-ATA</td>
<td>1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Tyr-GTA</td>
<td>13</td>
<td>1</td>
<td>92.9</td>
</tr>
<tr>
<td>Cys-ACA</td>
<td>1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Trp-CCA</td>
<td>1</td>
<td>8</td>
<td>11.1</td>
</tr>
</tbody>
</table>

Figure 1.3: Simplified representation of tRNA splicing and ligation pathways in mammals. After splicing out the intron from a pre-tRNA molecule by the TSEN complex, ligation of the tRNA exons can occur via either the yeast-like pathway or the animal-pathway. In the yeast-like pathway CLP1 phosphorylates the 5' end of the 3' exon. Ligation of the two exons is performed by a yet unidentified enzyme. The animal-like pathway includes direct ligation by RTCB.
exon is phosphorylated by CLP1. A yet unidentified phosphotransferase and ligase join the two exons. In the animal-like pathway, the two exons are ligated in a single GTP-dependent reaction involving the enzyme RTCB [62].

Besides intron removal and ligation, tRNA molecules undergo various modifications before they can function in protein synthesis. Removal of the 5’ and 3’ leader, addition of the CCA tail on the 3’end and various nucleotide modifications as methylations are necessary for a tRNA to become functional [63]. Once all modifications on the tRNA molecule are done, it can be charged with the corresponding amino acid. This reaction is performed by aminoacyl tRNA synthetases. For each amino acid a tRNA synthetase exists, e.g. RARS for arginine. Some synthetases have a nuclear encoded synthetase functioning in the mitochondria, for example RARS2. After charging with an amino acid, the tRNA can function in protein synthesis in the ribosomes.

Since many genes involved in PCH have a function in tRNA processing, it seems plausible that impairments in this pathway play part in the pathogenesis of the disease. Considering the indispensability of tRNAs in protein synthesis, it is conceivable that this latter process is affected as well. The first steps are made in elucidating whether this is indeed the case and how defects in these processing give rise to such a specific phenotype remains unclear. This topic will be extensively discussed in Chapter 5.

CONCLUSIONS

Pontocerebellar hypoplasia is a rare autosomal recessive neurodegenerative disorder. In the vast majority of patients, onset of the disease is prenatal and brain hypoplasia and clinical symptoms are progressive. Brain MRI of PCH patients show pontine and cerebellar hypoplasia, with variable cortical and white matter involvement. In the last decade, the spectrum and classification of pontocerebellar hypoplasia has been expanding. Whereas in the 1990’s two subtypes were distinguished, we now classify ten subtypes of this disease. Each subtype has its distinct clinical, radiological and genetic features. Implementation and development of next generation sequencing methods gave rise to novel genes associated with PCH. Interestingly, the majority of genes associated with PCH can be linked to RNA metabolism and protein synthesis. A start is made in elucidating how these mutations influence gene function and how this can lead to PCH. Nonetheless, there is still a lot to be discovered.
AIM AND OUTLINE OF THIS THESIS

In this thesis I investigated the genetics of pontocerebellar hypoplasia on various levels: I have identified novel genes for this disease and I describe novel subtypes and genotype-phenotypes correlations. Next, I investigate the pathology of PCH: what cellular and molecular effects do PCH-related mutations have?

In Chapter 2 we confirm the presence of PCH subtype 7 as an isolated syndrome with a single genetic locus. Exome sequencing revealed two candidate genes for PCH7 – CLK2 and TOE1. We discuss how we found TOE1 as disease-causing gene, and we describe a cohort of eight families with this novel disease. In Chapter 3 we zoom in on EXOSC3, a gene that causes PCH1. This subtype turns out to have a much broader phenotype than previously assumed. However, within the broad spectrum of EXOSC3-related PCH, clear genotype-phenotype correlations can be made. We describe a cohort of twelve families with EXOSC3 mutations and show that the specific genetic mutation can predict disease progression. Chapter 4 describes our research on the CLP1 gene in various aspects: we identify mutations in CLP1 to be PCH-causing, we model the disease in zebrafish and we investigate the effect of the mutation on cell homeostasis. Chapter 5 discusses potential cellular mechanisms underlying PCH and provides directions for further studies.
REFERENCES


